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STEROID-PROTEIN INTERACTIONS

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The species specificity which had been observed in rat and human "transcortin" for the species specific corticosteroid has not been found to be a general phenomenon. The corticosteroid-binding proteins in the sera of various mammalian species belong to the alpha globulin as was demonstrated by the method of equilibrium paper electrophoresis. Electrophoretic studies showed that the "transcortin" containing fraction of rat serum migrates faster than albumin at pH values below 7, whereas at higher pH it behaves as an alpha globulin.

The α_1-acid glycoprotein (orosomucoid) has been prepared by a combination of precipitation and chromatographic procedures. The interaction of progesterone with this glycoprotein has been found to be highly dependent on temperature; the association constant decreases with increasing temperature. A strong dependency on pH has also been observed; the association constant is highest at pH 8 and decreases to about 1/20 of this value at pH 2.3. The number of binding sites for progesterone has been determined to be n = 1 at pH 7.4, 4°C; the free energy of binding has the relatively high value of -7.5 kilocalories per mole.

Partial removal of sialic acid from the orosomucoid preparations reduced the binding affinity for progesterone. In biological assay studies on the ligated uterus horn of the mouse it was observed that progesterone is inactivated by binding to the α_1-acid glycoprotein.

I.

It was indicated in last year's report that new laboratories were being constructed in a building on the main campus of the university. These facilities have been essentially completed during the period covered in the present report, and our group has moved into the new laboratories at the end of April, 1963. Animal rooms, animal operating facilities and workshop are still under construction. The location of our new laboratory, close to the main campus of the university, gives us easy access to the library of the science building. The distance from the downtown Medical School is approximately seven minutes by car.

II.

In the first part of this report, results are summarized which have been published during the report period and which are listed in the bibliography under No. 1 to 5. The major portion of these results has been obtained in cooperative work with members of the Army Medical Research Laboratory at Fort Knox, Kentucky. For this reason, no reprints have been submitted of work which has not been supported by this contract. The following abstracts describe the results obtained in these papers.

Abstract of Publication No. 1: The effect of spirolactones (SC-9420 and SC-8109) on aldosterone binding to serum proteins was studied and compared with the competitive effect of other steroids, in order to investigate the possibility that the antagonism of aldosterone by spirolactones may be mediated through a competition for protein binding sites. Aldosterone was found to interact more strongly with whole human serum than with albumin, at both 37° and 4° C. Relatively large concentrations of spirolactones were required to displace aldosterone from human serum proteins; similar displacement was obtained with small concentrations of cortisol or progesterone. Variations in the serum levels of endogenous 17-hydroxycorticosteroids, produced by suppression of adrenocortical secretion or by ACTH administration, were shown to affect aldosterone binding.

Abstract of Publication No. 2: Equilibration of serum of adrenalectomized rats with normal rat serum at 37° C. results in a corticosterone level of the adrenalectomized sera higher than the average of the two sera. Subsequent determination of the corticosteroid-binding activity shows higher values in the equilibrated sera of the adrenalectomized animals. Similar results have been obtained with hypophysectomized rats although the increase of corticosteroid-binding activity was less pronounced.

Multiple equilibrium dialysis studies demonstrate an increase of the serum corticosteroid-binding activity between one and two days after adrenalectomy, reaching a maximal value of about twice normal. As shown previously, the binding activity of rat serum towards corticosterone is higher than towards cortisol. Injection of massive doses of corticosterone into adrenalectomized rats reduces the corticosteroid-binding activity to about one fourth, i.e., the same value obtained in normal animals after injection of equal amounts of corticosterone.

These results can serve as a basis for the interpretation of increased biological half-time of corticosteroids administered to adrenalectomized animals. Electrophoretic studies demonstrate an enhanced α-globulin fraction in the serum of adrenalectomized rats. Concerning the mechanism of increased corticosteroid-binding activity, a rise in the course of a general increase of the synthesis of α-globulins, or a specific physiological regulation of the binding activity are discussed; no decision can presently be made between the alternatives.

Abstract of Publication No. 3: Thymus, brain and heart were removed from the rat, homogenized, and fractionated by differential centrifugation. The uptake of cortisol or corticosterone by the various fractions was studied using the multiple equilibrium dialysis method. Both steroids interact with all the fractions to form weak dissociable bonds: the uptake of corticosterone is generally greater than that of cortisol. If the interaction of these steroids is expressed in percent bound per mg of protein the "microsomal" and super-natant fractions show a greater binding than the "nuclear" and "mitochondrial" fraction. No correlation could be established between age of the rat and extent of binding of the two steroids by subcellular fractions of thymus, brain and heart. These results were supplemented by equilibrium dialysis studies on the interaction between cortisol or corticosterone and total homogenates of thymus, brain and heart of the rat. The thymus preparations did not show any preferential binding of these corticosteroids as compared to the brain or heart preparations.

Abstract of Publication No. 4: 1. A simplified method for isolation of kidney and liver cell membranes is reported. 2. Aldosterone is weakly bound to kidney subcellular fractions as demonstrated by the techniques of equilibrium fractionation and equilibrium dialysis. 3. The liver and kidney cell membranes studied were found to either take up the steroids according to their lipid solubility or to bind them in accordance with the polarity rule for steroid-protein interaction. 4. Spirolactone failed to displace aldosterone from the kidney cell membranes.

Abstract of Publication No. 5: Bio-assay of mixtures of progesterone and alpha-1 acid glycoprotein in mice under the conditions of the Hooker-Forbes test demonstrated loss of progestational activity, presumably caused by the relatively strong interaction between progesterone and the glycoprotein.

In Publication No. 6 work in our laboratory has been reviewed for the period from 1961, including unpublished recent results. Some of these are discussed in Part III of the present report.

III.

It had been found previously that the corticosteroid-binding protein in rat serum bound corticosterone more firmly than cortisol. Since corticosterone is the main corticosteroid of the rat, this seemed to be indicative of a species specificity. When we tested the corresponding behavior in human serum, we found indeed that in this case cortisol, the principal corticosteroid of human serum, was bound more firmly to the human transcortin system. In further testing whether this specificity of the transcortin system for the species'

own corticosteroid was a general phenomenon, the serum combining affinity (C) for corticosterone and cortisol was determined for a number of species. The combining affinity is defined as

$$C = \frac{S_{bound}}{S_{unbound} \times P} \text{ liter gm}^{-1} \qquad \text{(I)}$$

where S_{bound} and $S_{unbound}$ indicate the concentration of bound and unbound steroid, respectively, and P the protein concentration in gm per liter.

It is evident from Table 1 that the binding ratio of the two corticosteroids

(TABLE 1)

C-Values for Cortisol (F) and Corticosterone (B)

Species	F/B* in Blood	4°C		37°C	
		F	B	F	B
Human	10	0.92	0.77	0.32	0.39
Monkey	20	0.65	1.00	0.14	0.48
Rat	0.05	1.22	1.86	0.22	0.59
Rabbit	0.05	1.11	0.74	0.49	0.27
Sheep	20	0.27	0.25	0.17	0.18
Steer	1	0.29	0.26	0.15	0.13
Starling	-	1.34	1.24	0.20	0.26
Horse	-	0.51	0.26	0.26	0.17
Guinea Pig	-	1.20	0.79	0.17	0.15
Dog	1	0.30	0.23	0.13	0.13
Pig	-	0.16	0.16	0.09	0.08
Cat	5	0.16	0.22	0.07	0.10

* ratio of cortisol concentration / corticosterone concentration

has no general relationship to the ratio of these two adrenal hormones in the blood. A comparison of the pairs human-monkey, rat-rabbit and sheep-steer illustrates this clearly. Differences in the binding values of different species are considerable; there are also marked differences between strains and between individuals of supposedly the same strain. We have analyzed the sera of various species by the method of equilibrium paper electrophoresis and have found that the high affinity corticosteroid-binding proteins in the normal and adrenalectomized rat, in rabbit, beef and horse belong to the alpha-globulin components.

The continuous-flow paper electrophoretic technique was used to purify the corticosteroid-binding globulin of rat serum. The separated fractions were dialyzed against distilled water, lyophilized and their combining affinity to corticosterone determined by equilibrium dialysis at 37°C. Surprisingly, the highest corticosterone-binding activity was observed in front of the albumin peak, and not in the alpha globulin component as expected from the equilibrium paper electrophoresis studied. A similar experiment with human serum showed a normal location of the transcortin activity in the alpha-globulin region.

To explain this paradoxical behavior of rat serum which was consistently found in a number of experiments, the lyophilized fractions were subjected to paper-strip electrophoresis at various pH values. At pH 8.6, the fractions obtained from the front shoulder of the albumin peak and containing the highest "transcortin" activity, migrated as alpha-globulins; most of them were free of albumin. This result restored the accordance with the equilibrium paper electrophoresis experiments. Paper-electrophoretic analysis of the active fractions and of whole rat serum at pH 7.8, 7.0, 5.5 and 4.0 revealed that a reversal of the relative electrophoretic mobility of the "transcortin"-containing alpha-globulin fraction and rat serum albumin occurs at about pH 7. A similar electrophoretic behavior is known for the α_1-acid glycoprotein which becomes a prealbumin at pH values of approximately 7 and lower (7). In contrast, the transcortin-containing fraction isolated by continuous flow paper electrophoresis of human serum shows even at pH 4 an electrophoretic mobility slower than that of human albumin. The abnormal electrophoretic behavior of the "transcortin"-containing rat serum fraction on the paper curtain is interpreted as caused by a decrease of pH on the paper due to the low buffering capacity of the veronal buffer used (μ 0.02).

Although these results will have to be verified with the pure corticosteroid-binding globulins, they suggest characteristic differences in the physicochemical and biological properties of the "transcortin" systems of different species.

Concerning our second objective, the interaction of progesterone with the α_1-acid glycoprotein (orosomucoid), some results have been discussed recently at a symposium of the American Oil Chemists' Society devoted to work on binding of lipids by proteins (6). The α_1-acid glycoprotein is not unique for human serum; similar acidic glycoproteins have been observed as normal serum constituents in all mammalian species so far studied (8). The pure bovine orosomucoid (9) interacts also with progesterone (Table 2), however, with a considerably lower affinity than its human counterpart.

(TABLE 2)

Combining Affinity (C) of Progesterone with α_1-Acid Glycoprotein at pH 7.4, 4°C	
Species	C
Human	14.6; 14.3
Bovine	3.4; 3.9

The interaction between progesterone and human α_1-acid glycoprotein is highly dependent on temperature, the association constant decreasing with rising temperature (Table 3). In contrast, the dissociation of the progesterone-human serum albumin complex increases only slightly at higher temperatures as also seen in Table 3.

(TABLE 3)

Temperature Dependency of Progesterone binding to α_1-Acid Glycoprotein (Prep. I-117) and to Human Serum Albumin		
Temperature	C-value for α_1-AGP	C-value for HSA
4°	18.2	1.59
22°	14.8	-
24°	-	1.23
37°	7.4	0.88
45°	-	0.62
50°	3.3	-

It should be noted that the low binding affinities of orosomucoid and human albumin at the highest temperature values are not caused by denaturation effects; they have been found reversible in both cases.

The hydrogen ion concentration also has a strong influence on the progesterone-orosomucoid interactions. Table 4 shows that the highest binding affinity is obtained at pH 8, and that it declines steeply towards lower pH-values.

(TABLE 4)

pH Dependency of Progesterone Binding to α_1-Acid Glycoprotein (Prep. I-117)

pH	Buffer (0.05 M)	C-value
2.30	Citrate-Phosphate	0.87
3.00	" "	2.4
4.00	" "	5.4
5.00	" "	8.8
6.00	" "	10.3
7.00	" "	13.6
7.40	" "	15.9
8.00	" "	18.0
7.40	Phosphate	16.3
8.00	Ammediol	14.5
9.00	"	11.6
10.00	"	11.6

This is again in contrast to the progesterone-albumin interaction where the influence of pH is less pronounced, and where the binding affinity increases with increasing pH up to approximately pH 11 (10). The nature of the buffer appears to have an influence on the combining affinity.

The number of binding sites for progesterone in human α_1-acid glycoprotein was determined by equilibrium dialysis, using different concentrations of progesterone-4-C^{14} at constant protein concentration. Two samples of human orosomucoid were employed, prepared by a procedure based on the first precipitation step of Michon (11) and the chromatographic techniques of Bezkorovainy and Winzler (12). One of these two preparations (I-117) had essentially the full progesterone binding activity observed previously (13), the other one (I-103) had been deactivated to about 40% of its original binding activity as measured by the C-value. The data obtained by equilibrium dialysis at pH 7.4, 4°C, were evaluated by the graphic method of reciprocal plot, as well as by the Scatchard plot (14). Both procedures are based on the equation

$$r = \frac{n\,K\,(S)}{1 + K\,(S)} \qquad \text{(II)}$$

where r equals the number of bound steroid molecules per total number of protein molecules, n the number of binding sites for the steroid on each protein molecule, K the association constant, and (S) the molar concentration of unbound steroid molecules. The assumption is made that the n binding sites are equivalent and independent, all having the same association constant K. If both sides of equation (II) are inverted, one obtains

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK\,(S)} \qquad \text{(III)}$$

The validity of this equation (III) can be tested by plotting 1/r versus 1/(S); a straight line should be obtained. The intercept on the oridinate gives the value 1/n, and the slope of the curve equals 1/nK. Table 5 shows that an approximate value of n = 1 was obtained for the number of binding sites on the active preparation, I-117, whereas less than 0.4 binding sites were revealed per molecule of the deactivated glycoprotein (I-103). This would indicate that the "deactivation" process completely inactivates about 60% of the orosomucoid molecules; the remainder should then be expected to retain full binding affinity. The association constants, K, have been calculated by the reciprocal plot method of Klotz (14,15); Table 5 shows that they are within range of error identical.

(TABLE 5)

Interaction Between Progesterone And α_1-Acid Glycoprotein At pH 7.4, 4°C

Preparation		I-117	I-103
C-Value		13.9	5.3
n	(Klotz) (Scatchard)	0.90 0.82	0.33 0.36
Association Constant	(Klotz) (Scatchard)	$8.1 \times 10^5\ M^{-1}$ 9.2	$9.7 \times 10^5\ M^{-1}$ 8.1
Free Energy of Binding (For n = 1)		-7.5 kcal per mole	-7.5 kcal per mole

Similar values have been obtained by calculating the association constants by Scatchard's procedure (16). The free energy of binding for the binding site ($n = 1$) has the relatively high value of 7.5 Kcal/mole for both preparations. These results indicate that the reactions that lead to deactivation of the orosomucoid do not effect a general decrease of the binding affinity, but rather complete inactivation of a portion of the glycoprotein molecules, leaving the remainder intact. The cause of the deactivation will be further studied; no differences between the preparations I-103 and I-117 could be detected in the sedimentation constants and in paper-electrophoretic behavior.

The α_1-acid glycoprotein molecule is characterized by the comparatively high content of about 12% sialic acid (N-acetylneuraminic acid) (17). Partial removal of the sialic acid under the mild conditions of enzymatic hydrolysis resulted in a reduction of the binding activity for progesterone; neuraminidase from clostridium perfringens or from inactivated influenza virus was used in these experiments. It is not known whether the sialic acid is directly involved in the interaction with progesterone, or whether the removal of this constituent with its electronegative charge leads to conformational or other changes in the glycoprotein molecule which decrease the binding affinity for progesterone.

The biological activity of the hormone-protein complex is of considerable interest in any study of interactions between these components. In the case of the corticosteroid-binding globulin, it has been shown that complex formation with corticosteroid hormones causes inactivation <u>in vitro</u> and <u>in vivo</u> (18,19). The problem of biological activity of the progesterone-orosomucoid complex was studied utilizing the intra-uterine test procedure of Hooker and Forbes (20). This method appeared particularly suitable since the test solution is injected directly into a ligated segment of the uterus horn of the mouse; the steroid-glycoprotein mixture does not enter into general circulation so that various possibilities of interfering reactions of the two components with other body constituents are avoided. Such a topical test therefore permits a simpler interpretation than a systemic assay procedure.

In cooperation with Dr. T. R. Forbes it was found that the interaction with orosomucoid leads to loss of progestational activity of progesterone (5). Mixtures of progesterone and α_1-acid glycoprotein were inactive although they contained amounts of progesterone exceeding the threshold dosis for a positive test reaction (0.2 $m\mu g$ progesterone). Evidently, the presence of the glycoprotein in the assay solutions prevented the tests from being positive.

A. Publications during period covered by this report.

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